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IN THE U.S. PATENT AND TRADEMARK OFFICE

Inventor	**Zoltan GREFF et al**	
Patent App.	**10/030,436**	
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For	**2,3-BENZODIAZEPINE DERIVATIVES**	
Art Unit	**1624**	Examiner **COLEMAN, B**

Hon. Commissioner of Patents
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DECLARATION UNDER 37 CFR 1.132

I, Zoltan Greff, a citizen of Hungary, residing at Gyongvirag u. 8, H-1106 Budapest, Hungary, declare as follows:

THAT I have a number of years of experience in the preparation and testing of pharmaceutically active compounds in the treatment of neurodegenerative disorders;

THAT my full curriculum vitae may be attached hereto;

THAT I am an Applicant in U.S. Patent Application Serial No. 10/030,436 filed 21 March 2002 and directed to 2,3-BENZODIAZEPINE DERIVATIVES;

THAT in order to establish that the present application enables one "skilled in the art" to use the compounds of the Formula (I) to treat a wide variety of neurodegenerative diseases in mammals, including humans, I have assembled the following background information and have either personally conducted or supervised the carrying out of the following tests:

BACKGROUND INFORMATION

The Examiner states that the specification does not provide enablement for the treatment of neurodegenerative diseases, therefore she rejects claims 16-27 under 35 USC 112, first paragraph on the grounds that the disclosure in the specification is non-enabling to permit one to use the new Formula (I) compounds to treat many of the neurodegenerative diseases. The Examiner states that neurodegenerative diseases cover a broad range of disorders with different etiology. She further states that established treatment plans for many neurodegenerative diseases are not treated by administering compounds similar as those of the Formula (I) disclosed in the present application. As an example, she mentioned Alzheimer disease, which is treated by administering acetylcholinesterase inhibitors.

1. Neurodegenerative diseases in general

Glutamate is the most abundant excitatory neurotransmitter in the brain. Glutamate receptors are categorized into ionotropic and metabotropic glutamate receptors. Ligand-gated ionotropic glutamate receptors are ion channels allowing cation flow into the neurons, which are categorized into the subgroups of NMDA, AMPA and kainic acid receptors (Dingledine, R. et al. (1999) The glutamate receptor ion channels. Pharmacol. Rev. 51, 7-61]. Excessive stimulation of ionotropic glutamate receptors, including

AMPA receptors can cause neuronal degeneration and cell death called excitotoxicity whereby desensitization is delayed and calcium ion permeability is increased [Bennett, M.V. et al. (1996) The G1uR2 hypothesis: Calcium-ion permeable AMPA receptors in delayed neurodegeneration. Cold Spring Harb. Symp. Quart. Biol. 61, 373-384; A. Frandsen et at. (2003). AMPA receptor-mediated neurotoxicity: Role of calcium ions and desensitization. Neurochem. Res. 28(10) 1495-1499]. Delayed desensitization leads to excess sodium and calcium ion entry into the cells.

Intracellular calcium ion concentration has a key role in the initiation of both apoptosis and necrosis.

AMPA receptor antagonists with 2,3-benzodiazepine moieties have been repeatedly shown to produce strong neuroprotective effect in several brain ischaemia models in laboratory species. Further, a great number of neurodegenerative diseases exists with different etiology that can be alleviated by administration of AMPA receptor antagonists with 2,3-benzodiazepine basic structure alone or in combination with other pharmaceutical agents. It is also important that despite the diverse and mostly unknown etiology of the neurodegenerative diseases (e.g. Alzheimer's disease, Parkinson's disease, Huntington's disease, Creutzfeld-Jacob disease, ALS, multiple sclerosis etc.), excitotoxicity is one of the common pathways causing substantial damage in the particular CNS areas involved in the given neurodegenerative disorder, thus antagonists

or negative modulators or the excitatory neurotransmitter system could have beneficial effects in all such diseases.

2. Parkinson's disease

Parkinson's disease is a slowly progressing CNS disorder characterized by an accelerated loss of dopaminergic nerve cells in the substantia nigra due to an unknown neurodegenerative process. Symptoms appear first by the time the majority of dopaminergic neurons in the nigrostriatal system are already lost. The drawback of the currently applied therapy comprising administration of levodopa (and/or carbidopa) is that on the long term, most of the patients develop dyskinesia. Corticostriatal and thalamostriatal glutaminergic excitatory pathways stimulating both NMDA and AMPA receptors are also involved in the neuronal organization of movement and seem to contribute to levodopa-induced dyskinesia. Furthermore, ligand binding was increased in the putamen of parkinsonian patients experiencing motor complications compared to those who did not, suggesting that glutamate receptor supersensitivity in the putamen plays a role in the development of motor complications [Dingledine, F. et al. (1999) The glutamate receptor ion channels. Pharmacol. Rev. 51, 7-61].

The role of AMPA receptors in the development of motor complications in monkeys rendered parkinsonian was studied in details [Gallyas, F. et al. (1980) A reliable and sensitive method

to localize terminal degeneration and lysosomes in the central nervous system. Stain Technol. 55, 299-306). Study findings showed that LY300164, a selective, non-competitive AMPA receptor antagonist potentiated the effects of low-dose levodopa on motor activity and decreased levodopa-induced dyskinesia (Abrandm, G. et al. (2000). New non-competitive AMPA antagonists. Bioorg. Med. Chem 8 2127-2143]. These results suggested that non-competitive AMPA receptor antagonists could be useful in the treatment of Parkinson's disease by enhancing the antiparkinsonian effects of levodopa and decreasing dyskinesia [Konitsiotis, S. et al. (2000) AMPA receptor blockade improves levodopa-induces dyskinesia in MPTP monkeys. Neurology 54,1589-1595].

Results of a pilot study of 30 patients with Parkinson's disease conducted at six US institutes and Europe indicate that talampanel, the active enantiomer of a compound with 2,3-benzodiazepine structure, may have a major role in decreasing levodopa caused dyskinesia. Currently, the Parkinson Center of the Department of Neurology at the University of Miami (US) conducts a study on talampanel as a treatment for dyskinesia, [www.parkinson.org].

Furthermore, improvement of akinesia was observed in rats with bilateral substantia nigra pars compacts lesion after administration of AMPA antagonists [Stauch, S.B. et al., (1995) Centrally

administered AMPA antagonists increase locomotion in parkinsonian rats. J. Neural Transm. Park Dis. Dement. Sect. 9, 145-149].

3. Multiple sclerosis

Multiple sclerosis is an autoimmune disease of the central nervous system that results in progressive fall of sensory and motor functions due to destruction of the myelin sheet of axons, thus resulting in neuronal death. It has been shown that glutamate receptors including AMPA receptors are present in oligodendrocytes and these cells are highly sensitive to glutamate-induced excitotoxicity [Mature, C. et al. (2001) There is a link between excitotoxic oligodendroglial death and demyelinating diseases. Trends Neurosci. 24, 224-230]. The experimental method EAE (experimental autoimmune encephalomyelitis) induced in laboratory animals can be used as animal model for multiple sclerosis. It has been shown that daily treatment with an AMPA antagonist result in remarkable improvement of motor disability induced by EAE in rats and mice in a dose-dependent manner, rescuing as much as 60% of oligodendrocytes destroyed by EAE. Further, loss of motor neurons in the ventral horn of the lumbar spinal chord was attenuated [Stauch, S.B. et at. (1995) Centrally administered AMPA antagonists increase locomotion in parkinsonian rats. J. Neural Transm. Park Dis. Dement, Sect. 9, 145-149]. These results suggest that glutamate induced excitotoxicity mediated by AMPA receptors

contributed to oligodendrocyte loss and motor disability and indicate the usefulness of AMPA antagonists in order to reduce neurological disability [Moga D. et al. (2002) Parvalbumin-containing interneurons in rat hippocampus have an AMPA receptor profile suggestive of vulnerability to excitotoxicity. J. Chem. Neuroanat. 23, 249-253].

EXPERIMENTAL SECTION

4. Global cerebral ischaemia

Neuroprotective effects of AMPA antagonists according to the present invention were studied in transient global ischaemia model in gerbils. Transient global ischaemia of the brain induces delayed damage to neurons including hippocampal pyramidal cells in the CAI sub-field and hilar neurons in the dentate gyrus and the striatal caudate-putamen region. The mongolian gerbil is a suitable animal for this model since due to defective brain circulation, bilateral carotid occlusion produces complete forebrain ischaemia in this species.

4.1. Methods

Male mongolian gerbils (60-70 g) were subjected to global ischaemia via bilateral common carotid artery occlusion (BCO) for 3 minutes under ether anaesthesia. During surgery, the body temperature of the animals was kept at the individual preoperative levels.

The compounds of the invention were administered at 20 mg/kg dose intraperitoneally, 45 minutes after reperfusion.

Four days after surgery the animals were deeply anaesthetised with pentobarbital (60 mg/kg i.p.) and perfused through the heart by a fixative solution containing 0.1 % glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. Brains were removed, post-fixed in the same solution overnight and 60 μm thick coronal sections were prepared.

Alternate coronal sections of brains were stained by silver impregnation according to. Gallyas et al [details [Gallyas, F. et al. (1980) A reliable and sensitive method to localize terminal degeneration and lysosomes in the central nervous system. Stain Technol. 55, 299-306]. Neuronal damage in the hippocampal CAI region was scored from 0 to 6 as follows: (0) undamaged; (1) <10%; (2) 10-30%; (3) 30-50%; (4) 50-70%; (5) 70-90%; (6) >90% cell loss.

In case of asymmetrical damage in the two sides, the rating was assigned to the higher score. Neurons were considered to be irreversibly damaged when they were shrunken and strongly argylophilic, somata of intact neurons appeared dark yellow. Differences among groups were statistically evaluated by Kruskal-Wallis ANOVA followed by Mann-Whitney U-test.

4.2. Results

Table 1 summarizes the neuroprotective effect of the compounds according to the present invention. According to results summarized in Table 1, compounds according to the present invention protected against neuronal death in the CAI area of the hippocampus of gerbils subjected to 3 min global cerebral ischaemia. These results suggest that compounds of the present invention can be suitable for the treatment of neurological consequences of global cerebral ischaemia, occurring in hypotension (blood loss), circulatory collapse during sever injury or due to cardiac arrest followed by resuscitation, cardiac surgery with extra-corporeal circulation etc.

Table 1

Neuroprotective effects of the compounds of the Formula (I) according the present invention (20 mg/kg i.p.) in the CAI area of mongolian gerbils subjected to 3 min bilateral carotid occlusion

Compound	Reduction in cal pyramidal cell death at day 4 after BCO
Example 27	51 %**
Example 28	45 %*
Example 30	47 %

** *p<0.05; ** p<0.01

5. Amyotrophic lateral sclerosis

The neuroprotective effects of the compounds of the invention in amyotrophic lateral sclerosis (ALS, motoneuron disease) was studied in cultured rat motor neurons against domoic acid induced neuronal death in vitro.

ALS is a severe neurodegenerative condition leading to relatively selective destruction of cortical, bulbar and spinal motor neurons. Glutamate induced excitotoxicity is one of the mechanisms contributing to the development of motoneuron disease.

5.1. Methods

Motoneurons were purified using a combination of density gradient centrifugation and immune-purification according to Henderson [Henderson at al. (1995). Survival of newly postmitotic motoneurons is transiently independent of exogenous trophic support. J-Neurosci. 15(4), 3128-37]. Spinal cords were dissected from day E14.5 Sprague- Dawley rat embrios (Elevage Janvier, France). The largest cells were isolated by centrifugation on a 6.5 % (w/v) metrizamide density gradient. The immonoaffinity purification step used previously was replaced by a cell sorting step using microbeads (Arce V. et al. (1999) Cardiotrophin-1 requires LIFRbeta to promote survival of mouse motoneurons purified by a novel technique [.J. Neurosci-Res. 55(1) 119-26]. Cells were incubated with a mouse antibody (anti-rat p75 antibody [Ig192)).

Subsequently motoneurons were incubated with magnetic microbeads conjugated to anti-mouse secondary antibodies, thus allowing the purification of motoneurons on separating columns (Miltenyi Biotech Inc.). Cells were centrifuged through a BSA cushion and resuspended in complete medium (neurobasal medium supplemented with B27 (Life Technologies), 2% horse serum, 25 Oil 2-mercaptoethanol. Cells were plated onto 384-well dishes coated with polyornithine and laminin in complete medium. Final volume was 100 μl in each well in the presence of 1 ng/ml brain-derived neurotropic factor (BDNF, R&D Systems).

After four days of culture (time for the upregulation of AMPA-kainate type glutamate receptors as the neurons mature) half of the medium was removed and replaced by a freshly made medium containing compounds. One hour later the cells were treated with domoic acid (10 μM final). Domoic acid, an AMPA-kainate receptor agonist was used instead of glutamate as it elicits non-desensitizing responses at AMPA receptors. The concentration of the domoic acid was optimized in preliminary experiments. Controls included (all with BDNF) no treatment, domoic acid treatment (10 μM final), domoic acid together with the compound of Example 27 according to the present invention at different concentrations. Eight replicates were performed for each settings. 50 μ l of the freshly prepared solutions were added to each well. Equal volume of the appropriate diluent was added to the controls.

The number of surviving motoneurons was counted 2 day later. Live cells were counted by an automated image analyzer (Trophos) after labelling with a vital dye, calcine-AM (Fluke). Results were analyzed using Student t-test (two-tailed, unpaired).

5.2. Results

The results of the study are summarized in Table 2. According to these results, the compound of Example 27 according to the present invention protected against domoic-acid induced cell death in cultured rat motoneurones in a concentration-dependent manner. It produced marked effect at the concentration of 10 μM suggesting that compounds according to the present invention can be suitable for the treatment of ALS.

Table 2. Survival of cultured rat motor neurons after domoic acid (DA induced cell death after treatment with the compound of Example 27 of the present invention

Treatment	Concentration of the compound of Example 27	Motor neuron survival (% of the control group)
(I M)ldomoic acid (10μM)		24
DA(10 μM)+Example 27	0.1	22
DA(10 μM)+Example 27	1	53
DA(10 iM)+Example 27	10	80***
DA(10 p.M)+Example 27	100	96***
*** p<0.001		

6. Stroke

Permanent or transient middle cerebral artery occlusion (MCAO) performed both in rats and mice is used as an animal model to mimic conditions that occur the in human brain during stroke.

6.1. MCAO in mice

6.1.1. Methods

Focal cortical ischaemia was produced by electrocoagulation of the left middle cerebral artery (MCA). Male MRI mice (30-35 g, Charles-River Hungary Ltd.) were anaesthetised with 2,2,2-tribromoethanol (500 mg/kg i.p., 10 mg/kg). An incision was made in the left ternporoparietal region of the head between the orbit and the ear. The temporal muscle was incised and reflected forward. A small burr hole was drilled into the lateral outer surface of the skull just over the MCA and the stem of MCA was occluded by electrocoagulation. Compounds were administered intraperitoneally 15 minutes before MCA occlusion. Two days later animals were anaesthetised deeply with pentobarbital (100 mg/kg i.p., 10 ml/kg), perfused through the heart with 4% solution of 2,3,5-triphenyltetrazolium chloride. Animals were decapitated, brains were removed and placed in saline containing 8% formaldehyde solution for 24 hours. The necrotic (non-stained) area was determined by means of image analysis (DigiCell for Windows 4.0). Results were expressed as means±SEM for the treatment groups and

statistical significance was assessed using ANOVA followed by Duncan test.

6.1.2. Results

Results are summarized in Table 3. According to these results, compounds of the present invention decrease cerebral infarct size after focal cerebral ischaemia in mice and produced strong neuroprotective effect with low minimum effective doses. Results suggest that the compounds of the Formula (I) of the present invention can be suitable for the treatment of human stroke.

Table 3.

Reduction of cerebral infarct size after focal cerebral ischaemia in mice

Compound	Minimum effective dose, mg/kg
Example 28	0.3
Example 34	3
Example 38	3

6.2. MCAO in rats

6.2.1. Methods

Permanent focal ischaemia was produced by electrocoagulation of the left MCA according to Brint et al. [Brint S. et al, (1988) Focal brain ischaemia in the rat: methods for reproducible neocortical infarction using tandem occlusion of the distal middle cerebral and ipsilateral common carotid arteries. J. Cereb. Blood Flow Metab. 8, 474-485]. Male Sprague Dawley rats (180-220 g) were anaesthetised with pentobarbital (60 mg/kg i.p.). The temporal muscle was incised anda 2 mm burr hole was drilled 2-3 mm rostal to the fusion of the zygomatic arch with the squamosal bone, exposing left MCA followed by MCA occlusion by electrocoagulation. The left common carotid artery was isolated and simultaneously occluded by bipolar electrocoagulation. Compounds were administered 30 minutes after MCA occlusion. After 48 hours, animals were deeply anaesthetised with pentobarbital (100 mg/kg perfused through the heart with 4% 2,3,5-triphenyltetrazolium chloride. Animals were decapitated, brains were removed and placed in saline containing 8% formaldehyde solution for at least 24 hours. Each brain was sliced into 1 mm sections including the necrotic tissue. Area of necrosis was measured in each slice using a morphometric software (DigiCell). From the area of infarcts, an estimate of the hemispheric extent of ischaemic damage expressed in volume units was calculated. Results were expressed as mean±SEM for the treatment

groups and statistical significance was assessed using ANOVA followed by Duncan test.

6.2.2. Results

It is concluded that compound of Example 28 decreased cerebral infarct size after focal cerebral ischaemia in rats and produced strong neuroprotective effect in an effective dose as low as 0.03 mg/kg and in a dose dependent manner. Results suggest that compounds according to the present invention could be suitable for the treatment of human stroke.

7. Cystic Periventricular Leukomalacia

Injection of S-bromo-willardiine or ibotenate on post-natal day 5 (P5) in mice induces neuronal death leading to cortical brain lesions that mimics several aspects of human cystic periventricular leukomalacia, which is observed most frequently in premature human infants [M, Largeron et al. (2001). The neuroprotective activity of 8-alkylamino-1,4-benzoxazine antioxidants is disclosed in Eur-J-Pharmacol. 424(3) 189-94].

7.1. Methods

At postnatal day 5, Swiss mouse pups were anaesthetised for intracerebral(i.c,) and intraperitoneal (i.p.) injections. I.c. injections were performed using a 26-gauge needle mounted on

calibrated microdispenser. The needle was inserted 2 mm under the external surface of scalp skin in the frontoparietal area of the right hemisphere 2 mm from the midline in the lateral-medial plane and 3 nun in the rostro-caudal plane from the juction between sagittal and lambdoid sutures. Two 1 μl boluses were injected at a 30 second interval. Fifteen micrograms of S-bromo-willardiine diluted in phosphate-buffered saline (PBS) was injected i.c.

Immediately following the i.c. injection of the excitotoxin, compound of example 27 of the present invention diluted in 5 μl PBS containing 10% dimethyl-sulfoxide (I7MSO) was administered intraperitoneally at a dose of 1, 3 or 10 mg/kg. Controls received the solvent (9:1 PBS-DMSO mixture) alone.

Five days later pups were sacrificed and brains fixed in formaldehyde solution. Coronal serial sections in 15 μm thickness were cut and each third section was stained with crasyl-violet. Brains were completely and serially sectioned from the frontal pole to the occipital lobes permitting an accurate and reproducible determination of the maximum sagittal frontooccipital diameter of both the cortical plate and white matter lesions. This parameter was used as a reliable and reproducible index of the lesion size, Statistical analyses were performed with Student t-test and one-way ANOVA. When group interaction was found to be significant, Dunnett multiple comparison test was performed. Results were expressed as

mean±SEM. All experimental protocols and procedures complied with guidelines of the INSERM and local ethical committees.

7.2. Results

It is shown that compound according to Example 27 of the present invention protected against S-bromo-willardiine induced lesions both in the cortical layers and the white matter in newborn mice at a dose of 1 mg/kg and 1 mg/kg, respectively and produced strong neuroprotective effect. The results suggest that the compounds of the Formula (I) according to the present invention can be successfully used for the treatment of human cystic periventricular leukomalacia.

THAT based upon the background information and experimental data presented above, I conclude the following:

The compounds of the Formula (I) of the present invention are non-competitive AMPA receptor antagonists. Experimental results obtained from animal model experiments indicate that compounds of the present invention possess strong neuroprotective effect, which is useful for the treatment of conditions and diseases of the central nervous system with seemingly very different etiology. In particular, the compounds of the Formula (I) of the present invention demonstrated good neuroprotective activity in generally accepted animal models of stroke, Parkinson's disease, multiple sclerosis, amyotrphic lateral sclerosis and cystic

periventricular leukomalacia, based on AMPA receptor antagonism. The mechanism whereby AMPA receptor antagonists prevent neuronal cell death in very different disorders is the inhibition of glutamate-induced excitotoxicity, which is a major mechanism leading to apoptosia and necrosis of nerve cells.

The experimental data presented hereinabove provide support for the establishment of utility of the compounds according to the present invention in several neurodegenerative disorders.

In summary, the compounds of the Formula (I) according to the present invention have surprisingly advantageous pharmacokinetic and metabolic properties which result in a preferable pharmacological and toxicological profile as well as in increased patient compliance.

THAT I am aware of no information inconsistent with that presented above or which would lead one to a contrary conclusion; and

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further

THAT these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Dated: _____ **Signed:** _____

Zoltan Greff